REMARKS

Upon entry of this response, claims 10-20 will be pending in this application. Claims 1-9 have been canceled. Claim 13 has been canceled as the recited feature (subcutaneous or intramuscular administration) is incorporated into claim 10. Amended claim 10 is fully supported by the specification at, for example, page 16, lines 10-12.

New claims 15-19 are fully supported by the specification at, for example, page 21, lines 1-9; page 11, lines 20-22, and page 29, Example 10. New claim 20 is fully supported by the specification at, for example, page 11, lines 4-8 and lines 14-19.

Amendment to the specification regarding the description of Figures 2A and 2B is fully supported by the specification at, for example, page 17, lines 10-14. Also, see the enclosed Habermann article (at page 52) that is referenced by the specification at page 15, lines 23-24. No new matter is added.

The Claims Are Definite

Claim 1-14 are rejected under 35 U.S.C. 112, second paragraph, for allegedly being indefinite because they lack the "essential steps" (method of administration, location, effective amount and/or outcome of the treatment).

The claims have been amended to recite these "essential steps". Thus, the claims are definite.

The Claims Are Novel

As will be explained in detail below, the claimed invention is novel because:

* a myofascial syndrome (also called a "localized fibromyalgia") is not a fibromyalgia

* administration of a botulinum toxin directly to a site of pain (i.e., "trigger point") is different from administration of a botulinum toxin to a "location anatomically distinct from" or "distant from" a site of pain to treat the pain.

The Borodic Reference

Claim 1-13 are rejected under 35 U.S.C. 102(b) for allegedly being anticipated by WO 94/15629 (hereinafter "the Borodic reference"). The Borodic reference cannot anticipate the claimed invention because the Borodic reference discloses a method of treating a "localized fibromyalgia", not the claimed fibromyalgia.

A "localized fibromyalgia" is not a fibromyalgia at all. In other words, a "localized fibromyalgia" is not a fibromyalgia, but is a myofascial syndrome. In fact, the Borodic reference clearly states that a "localized fibromyalgia" (i.e., a myofascial syndrome) is a different condition from fibromyalgia:

> Myofascial syndrome sometimes is **misdiagnosed** as fibromyalgia or fibrositis syndrome These terms in older literature may have been interchangeable with chronic muscular pain; therefore, myofascial pain sometimes can be viewed as a localized fibromyalgia.
>
> Fibromyalgia is a generalized syndrome characterized by tenderness within diffused distribution of muscle groups and associated systemic complaints such as sleep disturbances, generalized fatigue, chronic headaches and irritable bowel symptoms. **These characteristics distinguish it from myofascial pain syndrome.**

The Borodic reference, paragraph bridging page 1 and 2, emphasis added.

Further, Alvarez et al. (American Family Physicians 65(4):653-660, hereinafter "the Alvarez reference, enclosed herewith) states that, "myofascial pain syndrome is a common painful muscle disorder caused by myofascial trigger points.[] This must be **differentiated** from fibromyalgia syndrome..." (the Alvarez reference, page 653, first column, first paragraph). (emphasis added).

Thus, contrary to the allegation of the Office Action, a "localized fibromyalgia" is not a fibromyalgia, but is a myofascial syndrome; and the Borodic reference does not disclose a method of treating fibromyalgia. Accordingly, the Borodic reference cannot anticipate the claimed invention.

The Paulson Reference

Claims 1, 2, 4-7 and 9 are rejected under 35 U.S.C. 102(b) as being anticipated by Paulson et al. (Movement Disorders 11, 459 (1996), hereinafter "the Paulson reference"). The Paulson reference cannot anticipate the claimed invention because the Paulson reference teaches the administration of a botulinum toxin to a site that is different from that of the claimed invention.

The claims recite that the site of administration is "anatomically distinct" from the site of pain relief. As defined by the specification, "anatomically distinct" means that the functional anatomy of the first and second locations is not contiguous, in a functional sense (the Specification, page 12, lines 21-23). For example, the head and back are "anatomically distinct".

The Paulson reference, on the other hand, teaches the administration of the botulinum toxin to the same anatomical body part of where the pain is. For example, the Paulson reference teaches the administration of a botulinum toxin directly to a "trigger point" across the trapezius muscle (i.e., to the shoulder muscle) to treat a pain on the shoulder. Clearly the injection site of the botulinum toxin and the site of pain relief are not anatomically distinct, as they are both on the shoulder.

Thus, the claimed invention is novel over the Paulson reference.

The Asherson Reference

Claims 1-9 are rejected under 35 U.S.C. 102(a) as being anticipated by Asherson et al. (J. Rheumatol 28(7), 1740 (1996), hereinafter "the Asherson reference"). The

Asherson reference cannot anticipate the claimed invention because the Asherson reference does not teach an administration of a botulinum toxin to an anatomically distinct body part of where the pain is.

For example, the Asherson reference teaches the administration of the botulinum toxin directly to a "trigger point" (Office Action, page 4). However, the Asherson reference does not teach of the location of pain alleviation after an administration of botulinum toxin to a trigger point. The Asherson reference merely discloses that fibromyalgia patients have trigger points affecting the cervical area, upper shoulders, borders of the scapulae, and lower back, as well as medial aspect of knees, and that botulinum toxin injections into trigger points "offer more prolonged relief". However, the Asherson reference does not teach that the trigger points and location of pain are at anatomically distinct locations.

At most, the Asherson reference teaches an administration of a botulinum toxin **directly to the site of pain** ("trigger point") to treat the pain. For example, it is well known that a trigger point is a site of pain. Thus, the injection of botulinum toxin to the trigger points alleviates pain for these patients at these trigger points. However, the claimed invention recites that the botulinum toxin is administered to a location that is "anatomically distinct from" and/or "anatomically distant from" the location of pain (i.e., "second location") to treat the pain. In other words, the claimed invention excludes the step of administering a botulinum toxin directly to the site of pain to treat the pain.

Thus, the claimed invention is novel over the Asherson reference.

Terminal Disclaimer

Claims 1-14 are rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-32 of U.S. Patent No. 6,623,742 (hereinafter "the 742 patent").

DOCKET NO.: ALLE0049-100 (17455 CIP1) **PATENT**
U.S. Serial No. 10/666,408

Applicant hereby submits a terminal disclaimer under 37 C.F.R. 1.1321(c) with respect to the 742 patent for claims 1-14 of the present application. Thus, the obviousness-type double patenting rejection should be withdrawn. The enclosed terminal disclaimer is unexecuted, an executed terminal disclaimer will be mailed shortly.

Further, Applicant asserts that the terminal disclaimer is submitted for the sole purpose of administrative efficiency, and Applicant respectfully disagrees that the pending claims are obvious over the 742 patent. (The filing of a terminal disclaimer to obviate a rejection based on nonstatutory double patenting is not an admission of the propriety of the rejection. *Quad Environmental Technologies Corp. v. Union Sanitary District*, 946 F.2d 870, 20 USPQ2d 1392 (Fed. Cir. 1991), MPEP §804.02 II).

In view of the foregoing, Applicant submits that the pending claims are in condition for allowance, and an early Office Action to that effect is earnestly solicited.

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Doc No. 2440440

Trigger Points: Diagnosis and Management

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Trigger points are discrete, focal, hyperirritable spots located in a taut band of skeletal muscle. They produce pain locally and in a referred pattern and often accompany chronic musculoskeletal disorders. Acute trauma or repetitive microtrauma may lead to the development of stress on muscle fibers and the formation of trigger points. Patients may have regional, persistent pain resulting in a decreased range of motion in the affected muscles. These include muscles used to maintain body posture, such as those in the neck, shoulders, and pelvic girdle. Trigger points may also manifest as tension headache, tinnitus, temporomandibular joint pain, decreased range of motion in the legs, and low back pain. Palpation of a hypersensitive bundle or nodule of muscle fiber of harder than normal consistency is the physical finding typically associated with a trigger point. Palpation of the trigger point will elicit pain directly over the affected area and/or cause radiation of pain toward a zone of reference and a local twitch response. Various modalities, such as the Spray and Stretch technique, ultrasonography, manipulative therapy and injection, are used to inactivate trigger points. Trigger-point injection has been shown to be one of the most effective treatment modalities to inactivate trigger points and provide prompt relief of symptoms. (Am Fam Physician 2002;65:653-60. Copyright© 2002 American Academy of Family Physicians.)

Members of various medical faculties develop articles for "Practical Therapeutics." This article is one in a series coordinated by the Department of Family Medicine at the University of Michigan Medical School, Ann Arbor. Guest editor of the series is Barbara S. Apgar, M.D., M.S., who is also an associate editor of AFP.

About 23 million persons, or 10 percent of the U.S. population, have one or more chronic disorders of the musculoskeletal system.[1] Musculoskeletal disorders are the main cause of disability in the working-age population and are among the leading causes of disability in other age groups.[2] Myofascial pain syndrome is a common painful muscle disorder caused by myofascial trigger points.[3] This must be differentiated from fibromyalgia syndrome, which involves multiple tender spots or tender points.[3] These pain syndromes are often concomitant and may interact with one another.

Trigger points are discrete, focal, hyperirritable spots located in a taut band of skeletal muscle. The spots are painful on compression and can produce referred pain, referred tenderness, motor dysfunction, and autonomic phenomena.[4]

TABLE 1
Trigger Points vs. Tender Points

Trigger points	*Tender points*
Local tenderness, taut band, local twitch response, jump sign	Local tenderness
Singular or multiple	Multiple
May occur in any skeletal muscle	Occur in specific locations that are symmetrically located
May cause a specific referred pain pattern	Do not cause referred pain, but often cause a total body increase in pain sensitivity

Trigger points are classified as being active or latent, depending on their clinical characteristics.[5] An active trigger point causes pain at rest. It is tender to palpation with a referred pain pattern that is similar to the patient's pain complaint.[3,5,6] This referred pain is felt not at the site of the trigger-point origin, but remote from it. The pain is often described as spreading or radiating.[7] Referred pain is an important characteristic of a trigger point. It differentiates a trigger point from a tender point, which is associated with pain at the site of palpation only *(Table 1)*.[8]

A latent trigger point does not cause spontaneous pain, but may restrict movement or cause muscle weakness.[6] The patient presenting with muscle restrictions or weakness may become aware of pain originating from a latent trigger point only when pressure is applied directly over the point.[9]

Moreover, when firm pressure is applied over the trigger point in a snapping fashion perpendicular to the muscle, a "local twitch response" is often elicited.[10] A local twitch response is defined as a transient visible or palpable contraction or dimpling of the mus-

cle and skin as the tense muscle fibers (taut band) of the trigger point contract when pressure is applied. This response is elicited by a sudden change of pressure on the trigger point by needle penetration into the trigger point or by transverse snapping palpation of the trigger point across the direction of the taut band of muscle fibers. Thus, a classic trigger point is defined as the presence of discrete focal tenderness located in a palpable taut band of skeletal muscle, which produces both referred regional pain (zone of reference) and a local twitch response. Trigger points help define myofascial pain syndromes.

Tender points, by comparison, are associated with pain at the site of palpation only, are not associated with referred pain, and occur in the insertion zone of muscles, not in taut bands in the muscle belly.[8] Patients with fibromyalgia have tender points by definition. Concomitantly, patients may also have trigger points with myofascial pain syndrome. Thus, these two pain syndromes may overlap in symptoms and be difficult to differentiate without a thorough examination by a skilled physician.

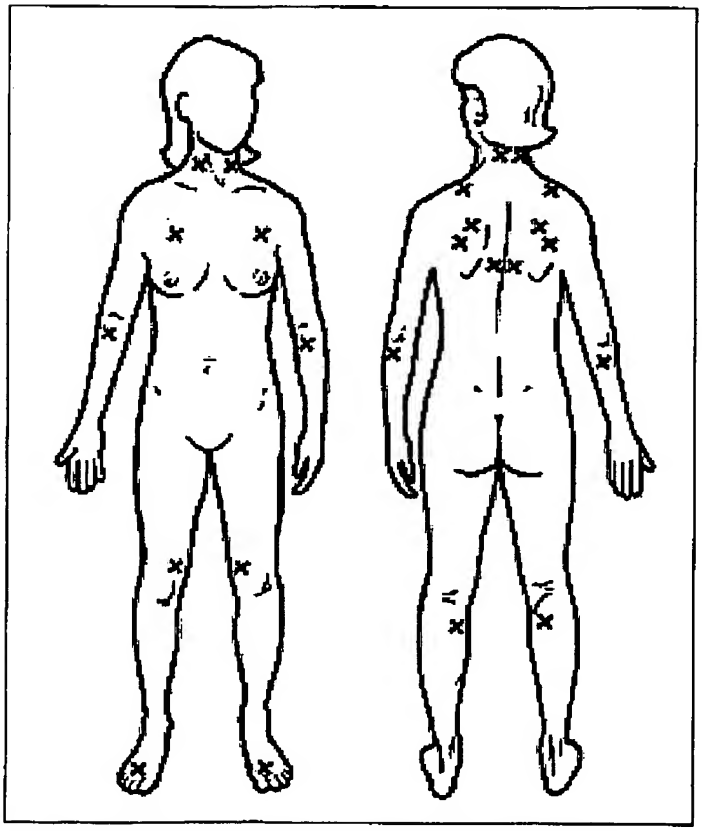

FIGURE 1. Most frequent locations of myofascial trigger points.

Pathogenesis

There are several proposed histopathologic mechanisms to account for the development of trigger points and subsequent pain patterns, but scientific evidence is lacking. Many researchers agree that acute trauma or repetitive microtrauma may lead to the development of a trigger point. Lack of exercise, prolonged poor posture, vitamin deficiencies, sleep disturbances, and joint problems may all predispose to the development of microtrauma.[5] Occupational or recreational activities that produce repetitive stress on a specific muscle or muscle group commonly cause chronic stress in muscle fibers, leading to trigger points. Examples of predisposing activities include holding a telephone receiver between the ear and shoulder to free arms; prolonged bending over a table; sitting in chairs with poor back support, improper height of arm rests or none at all; and moving boxes using improper body mechanics.[11]

Acute sports injuries caused by acute sprain or repetitive stress (e.g., pitcher's or tennis elbow, golf shoulder), surgical scars, and tissues under tension frequently found after spinal surgery and hip replacement may also predispose a patient to the development of trigger points.[12]

Clinical Presentation

Patients who have trigger points often report regional, persistent pain that usually results in a decreased range of motion of the muscle in question. Often, the muscles used to maintain body posture are affected, namely

the muscles in the neck, shoulders, and pelvic girdle, including the upper trapezius, scalene, sternocleidomastoid, levator scapulae, and quadratus lumborum.[13] Although the pain is usually related to muscle activity, it may be constant. It is reproducible and does not follow a dermatomal or nerve root distribution. Patients report few systemic symptoms, and associated signs such as joint swelling and neurologic deficits are generally absent on physical examination.[14]

In the head and neck region, myofascial pain syndrome with trigger points can manifest as tension headache, tinnitus, temporomandibular joint pain, eye symptoms, and torticollis.[15] Upper limb pain is often referred and pain in the shoulders may resemble visceral pain or mimic tendonitis and bursitis.[5,16] In the lower extremities, trigger points may involve pain in the quadriceps and calf muscles and may lead to a limited range of motion in the knee and ankle. Trigger-point hypersensitivity in the gluteus maximus and gluteus medius often produces intense pain in the low back region.[15] Examples of trigger-point locations are illustrated in *Figure 1*.[16]

Evaluation

Palpation of a hypersensitive bundle or nodule of muscle fiber of harder than normal consistency is the physical finding most often associated with a trigger point.[10] Localization of a trigger point is based on the physician's sense of feel, assisted by patient expressions of pain and by visual and palpable observations of local twitch response.[10] This palpation will elicit pain over the palpated muscle and/or cause radiation of pain toward the zone of reference in addition to a twitch response. The commonly encountered locations of trigger points and their pain reference zones are consistent.[8] Many of these sites and zones of referred pain have been illustrated in *Figure 2*.[10]

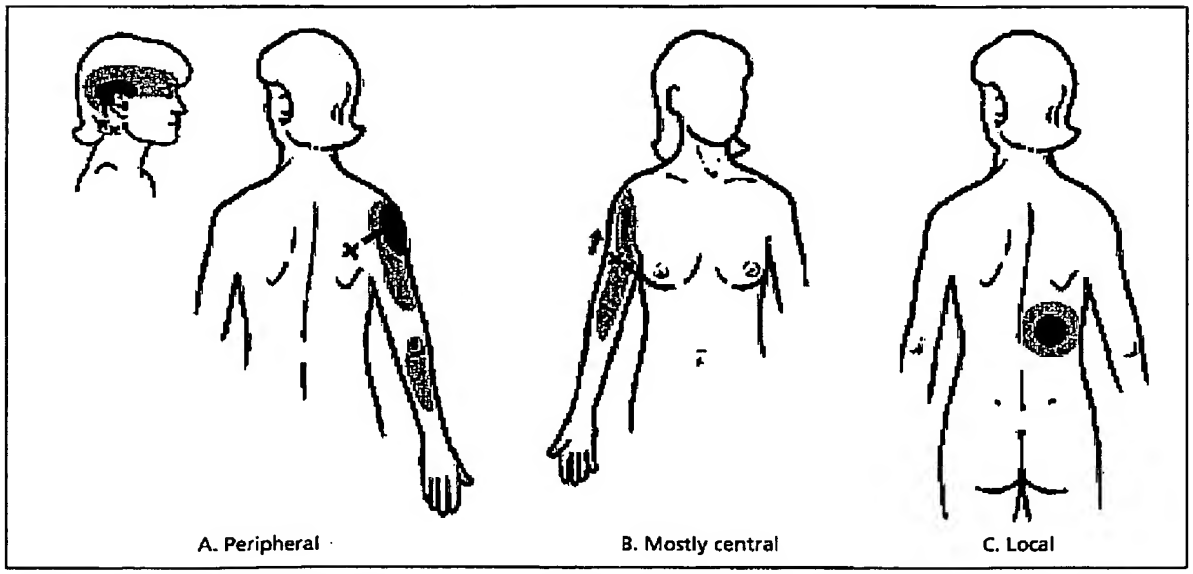

FIGURE 2. Examples of the three directions in which trigger points *(Xs)* may refer pain *(red)*. *(A)* Peripheral projection of pain from suboccipital and infraspinatus trigger points. *(B)* Mostly central projection of pain from biceps brachii trigger points with some pain in the region of the distal tendinous attachment of the muscle. *(C)* Local pain from a trigger point in the serratus posterior inferior muscle.

No specific laboratory test, imaging study, or interventional modality such as electromyography or muscle biopsy has been established for diagnosing trigger points.

No laboratory test or imaging technique has been established for diagnosing trigger points.[9] However, the use of ultrasonography, electromyography, thermography, and muscle biopsy has been studied.

Management

Predisposing and perpetuating factors in chronic overuse or stress injury on muscles must be eliminated, if possible. Pharmacologic treatment of patients with chronic musculoskeletal pain includes analgesics and medications to induce sleep and relax muscles. Antidepressants, neuroleptics, or nonsteroidal anti-inflammatory drugs are often prescribed for these patients.[1]

Nonpharmacologic treatment modalities include acupuncture, osteopathic manual medicine techniques, massage, acupressure, ultrasonography, application of heat or ice, diathermy, transcutaneous electrical nerve stimulation, ethyl chloride Spray and Stretch technique, dry needling, and trigger-point injections with local anesthetic, saline, or steroid. The long-term clinical efficacy of various therapies is not clear, because data that incorporate pre- and post-treatment assessments with control groups are not available.

The Spray and Stretch technique involves passively stretching the target muscle while simultaneously applying dichlorodifluoromethane-trichloromonofluoromethane (Fluori-Methane) or ethyl chloride spray topically.[5] The sudden drop in skin temperature is thought to produce temporary anesthesia by blocking the spinal stretch reflex and the sensation of pain at a higher center.[5,10] The decreased pain sensation allows the muscle to be passively stretched toward normal length, which then helps to inactivate trigger points, relieve muscle spasm, and reduce referred pain.[5]

Dichlorodifluoromethane-trichloromonofluoromethane is a nontoxic, nonflammable vapor coolant spray that does not irritate the skin but is no longer commercially available for other purposes because of its effect in reducing the ozone layer. However, its use is safer for both patient and physician than the original volatile vapor coolant, ethyl chloride. Ethyl chloride is a rapid-acting general anesthetic that becomes flammable and explosive when 4 to 15 percent of the vapor is mixed with air.[10] Nevertheless, ethyl chloride remains a popular agent because of its local anesthetic action and its greater cooling effect than that of dichlorodifluoromethane-trichloromonofluoromethane.[5]

The decision to treat trigger points by manual methods or by injection depends strongly on the training and skill of the physician as well as the nature of the trigger point itself.[10] For trigger points in the acute stage of formation (before additional pathologic changes develop), effective treatment may be delivered through physical therapy. Furthermore, manual methods are indicated for patients who have an extreme fear of needles or when the trigger point is in the middle of a muscle belly

TABLE 2
Equipment Needed for Trigger-Point Injection

Rubber gloves
Gauze pads
Alcohol pads for cleansing skin
3- or 5-mL syringe
Lidocaine (Xylocaine, 1 percent, without epinephrine) or procaine (Novocain, 1 percent)
22-, 25-, or 27-gauge needles of varying lengths, depending on the site to be injected
Adhesive bandage

Information from references 10 and 18.

TABLE 3
Contraindications to Trigger-Point Injection

Anticoagulation or bleeding disorders
Aspirin ingestion within three days of injection
The presence of local or systemic infection
Allergy to anesthetic agents
Acute muscle trauma
Extreme fear of needles

Information from references 10 and 18.

not easily accessible by injection (i.e., psoas and iliacus muscles).[10] The goal of manual therapy is to train the patient to effectively self-manage the pain and dysfunction. However, manual methods are more likely to require several treatments and the benefits may not be as fully apparent for a day or two when compared with injection.[10]

While relatively few controlled studies on trigger-point injection have been conducted, trigger-point injection and dry needling of trigger points have become widely accepted. This therapeutic approach is one of the most effective treatment options available and is cited repeatedly as a way to achieve the best results.[5]

Trigger-point injection is indicated for patients who have symptomatic active trigger points that produce a twitch response to pressure and create a pattern of referred pain. In comparative studies,[17] dry needling was found to be as effective as injecting an anesthetic solution such as procaine (Novocain) or lidocaine (Xylocaine).[10] However, postinjection soreness resulting from dry needling was found to be more intense and of longer duration than the soreness experienced by patients injected with lidocaine.[10]

One noncontrolled study[17] comparing the use of dry needling versus injection of lidocaine to treat trigger points showed that 58 percent of patients reported complete relief of pain immediately after trigger-point injection and the remaining 42 percent of patients claimed that their pain was minimal (1-2/10) on the pain scale. Both dry needling and injection with 0.5 percent lidocaine were equally successful in reducing myofascial pain. Postinjection soreness, a different entity than myofascial pain, often developed, especially after use of the dry needling technique.[17] These results support the opinion of most researchers that the critical therapeutic factor in both dry needling and injection is mechanical disruption by the needle.[1,10]

Injection with lidocaine has been shown to be effective in patients who have symptomatic active trigger points that produce a twitch response to pressure and create a pattern of referred pain.

TECHNIQUE OF TRIGGER-POINT INJECTION

Trigger-point injection can effectively inactivate trigger points and provide prompt, symptomatic relief. *Table 2*[10,18] outlines the necessary equipment for trigger-point injection. Contraindications to trigger-point injection are listed in *Table 3*[10,18] and possible complications are outlined in *Table 4*.

Preinjection. Increased bleeding tendencies should be explored before injection. Capillary hemorrhage augments postinjection soreness and leads to unsightly ecchymosis.[10] Patients

TABLE 4
Complications of Trigger-Point Injections

Vasovagal syncope
Skin infection
Pneumothorax; avoid pneumothorax complications by never aiming a needle at an intercostal space.
Needle breakage; avoid by never inserting the needle to its hub.
Hematoma formation; avoid by applying direct pressure for at least two minutes after injection.

should refrain from daily aspirin dosing for at least three days before injection to avoid increased bleeding.

The patient should be placed in a comfortable or recumbent position to produce muscle relaxation. This is best achieved by positioning the patient in the prone or supine position. This positioning may also help the patient to avoid injury if he or she has a vasovagal reaction.[18]

Needle Selection. The choice of needle size depends on the location of the muscle being injected. The needle must be long enough to reach the contraction knots in the trigger point to disrupt them. A 22-gauge, 1.5-inch needle is usually adequate to reach most superficial muscles. For thick subcutaneous muscles such as the gluteus maximus or paraspinal muscles in persons who are not obese, a 21-gauge, 2.0-inch needle is usually necessary.[10] A 21-gauge, 2.5-inch needle is required to reach the deepest muscles, such as the gluteus minimus and quadratus lumborum, and is available as a hypodermic needle. Using a needle with a smaller diameter may cause less discomfort; however, it may provide neither the required mechanical disruption of the trigger point nor adequate sensitivity to the physician when penetrating the overlying skin and subcutaneous tissue. A needle with a smaller gauge may also be deflected away from a very taut muscular band, thus preventing penetration of the trigger point. The needle should be long enough so that it never has to be inserted all the way to its hub, because the hub is the weakest part of the needle and breakage beneath the skin could occur.[6]

Injection Solutions. An injectable solution of 1 percent lidocaine or 1 percent procaine is usually used. Several other substances, including diclofenac (Voltaren), botulinum toxin type A (Botox), and corticosteroids, have been used in trigger-point injections. However, these substances have been associated with significant myotoxicity.[10,19] Procaine has the distinction of being the least myotoxic of all local injectable anesthetics.[10]

Injection Technique. Once a trigger point has been located and the overlying skin has been cleansed with alcohol, the clinician isolates that point with a pinch between the thumb and index finger or between the index and middle finger, whichever is most comfortable *(Figures 3a and 3b).* Using sterile technique, the needle is then inserted 1 to 2 cm away from the trigger point so that the needle may be advanced into the trigger point at an acute angle of 30 degrees to the skin. The stabilizing fingers apply pressure on either side of the injection site, ensuring adequate tension of the muscle fibers to allow penetration of the trigger point but preventing it from rolling away from the advancing needle.[10] The application of pressure also helps to prevent bleeding within the subcutaneous tissues and the subsequent irritation to the muscle that the bleeding may produce. The serious complication of pneumothorax can be avoided by refraining from aiming the needle at an intercostal space.

Before advancing the needle into the trigger point, the physician should warn the patient of the possibility of sharp pain, muscle twitching, or an unpleasant sensation as the needle contacts the taut muscular band.[17] To ensure that the needle is not within a blood vessel, the plunger should be withdrawn before

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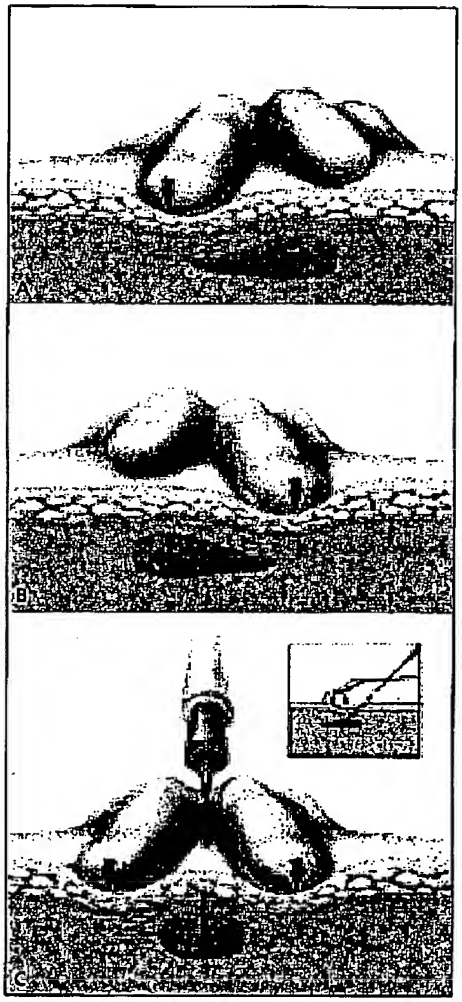

FIGURE 3. Cross-sectional schematic drawing of flat palpation to localize and hold the trigger point *(dark red spot)* for injection. *(A, B)* Use of alternating pressure between two fingers to confirm the location of the palpable nodule of the trigger point. *(C)* Positioning of the trigger point halfway between the fingers to keep it from sliding to one side during the injection. Injection is away from fingers, which have pinned down the trigger point so that it cannot slide away from the needle. Dotted outline indicates additional probing to explore for additional adjacent trigger points. The fingers are pressing downward and apart to maintain pressure for hemostasis.

The needle should be inserted 1 to 2 cm away from the trigger point and slowly advanced at a 30-degree angle to the skin into the area of pain.

injection. A small amount (0.2 mL) of anesthetic should be injected once the needle is inside the trigger point. The needle is then withdrawn to the level of the subcutaneous tissue, then redirected superiorly, inferiorly, laterally and medially, repeating the needling and injection process in each direction until the local twitch response is no longer elicited or resisting muscle tautness is no longer perceived *(Figure 3c)*.[10]

Post-injection Management. After injection, the area should be palpated to ensure that no other tender points exist. If additional tender points are palpable, they should be isolated, needled and injected. Pressure is then applied to the injected area for two minutes to promote hemostasis.[10] A simple adhesive bandage is usually adequate for skin coverage.

One study[20] emphasizes that stretching the affected muscle group immediately after injection further increases the efficacy of trigger point therapy. Travell recommends that this is best performed by immediately having the patient actively move each injected muscle through its full range of motion three times, reaching its fully shortened and its fully lengthened position during each cycle.[10]

Postinjection soreness is to be expected in most cases, and the patient's stated relief of the referred pain pattern notes the success of the injection. Re-evaluation of the injected areas may be necessary, but reinjection of the trigger points is not recommended until the postinjection soreness resolves, usually after three to four days. Repeated injections in a particular muscle are not recommended if two or three previous attempts have been unsuccessful. Patients are encouraged to remain active, putting muscles through their full range of motion in the week following

trigger-point injections, but are advised to avoid strenuous activity, especially in the first three to four days after injection.[10]

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^{125}I-Labeled Neurotoxin from Clostridium Botulinum A: Preparation, Binding to Synaptosomes and Ascent to the Spinal Cord

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Summary. 1. Labeling of crystalline botulinum A toxin has been done with ^{131}I by aid of the chloramine T method. The neurotoxic component is well preserved, whereas the hemagglutinin undergoes physicochemical alterations. Neither with labeled nor with unlabeled toxin, hemagglutinating power parallels the main protein peak.

2. Neurotoxin, homogeneous in gel filtration, is bound to synaptosomes from rat brain. Cold toxin competes with labeled toxin, and antitoxin or neuraminidase partially remove the bound neurotoxin.

3. Upon intramuscular injection, some radioactivity is recovered in the respective parts of the spinal cord. Antitoxin prevents the ascent.

The similarities between tetanus and botulinum A neurotoxins are stressed.

Key words: Botulinum A — Tetanus — Neurotoxin — Hemagglutinin — Iodine Labeling.

The neurotoxins of clostridium botulinum A and clostridium tetani share many properties. Their molecular weights are in the same range, their actions at the peripheral neuromuscular synapses are quite similar, their effects last extremely long, and they are the most potent toxins ever found. They differ by the predominance of peripheral actions in botulism whereas tetanus toxin exerts its effects, under the usual conditions, at the central level (see Mellanby *et al.*, 1973). The question whether botulinum toxin can be fixed to CNS matter, and especially to gangliosides, is still controversial. In contrast to Simpson and Rapport (1971a, b), Van Heyningen and Mellanby (1973) and Mellanby *et al.* (1973) failed to observe an inactivation by gangliosides or ganglioside-cerebroside complexes, and Coleman (1924) could not reproduce the Wassermann-Takaki-phenomenon with botulinum toxin, i.e. the fixation by brain matter.

Previous investigations in this laboratory (see Habermann and Lembeck, 1973) shed new light on the pharmacodynamics and pharmacokinetics of tetanus toxin. Botulinum toxin was included into these

studies because we expected new insights into its mode of action by aid of the techniques developed for tetanus toxin.

Material and Methods

Botulinum Toxin. Two samples, freeze-dried from crystalline toxin, have been obtained from Dr. E. J. Schantz, University of Wisconsin, Madison (U.S.A.). The first sample was kept for several years in the cold room and had its LD_{50} (mouse, s.c.) near 4.5 ng/kg. This toxin was used for pilot studies, and gave results indistinguishable from those with the second sample. The latter had been freshly prepared, and its LD_{50} was 3.0 ng/kg, which is close to that mentioned by Dr. Schantz (3.3×10^7 mouse LD_{50}/mg N). The toxicity was not altered when we froze the solution quickly with acetone-CO_2 and kept it for at least some months at −20°C. So we preserved the toxin by that way and thawed aliquots when needed.

Protein content was measured according to Lowry *et al.* (1951). When only very small amounts were at disposal, dilutions of toxin were excited at 285 nm and their fluorescence measured at 350 nm in a Aminco-Bowman spectrofluorimeter.

Toxicity was measured in NMRI mice, own breed, by subcutaneous injection into the neck region.—The neural ascent of toxin was studied in Wistar rats, own breed, weighing between 200 g and 250 g. Labeled toxin, equivalent to about 600 mouse-LD per 100 g rat, was injected into one m. gastrocnemius or one forelimb of rats. They were killed and dissected about 20 h later.

For *hemagglutination assays* dilutions (0.1 ml) of the toxin were made in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and mixed with the same volume of 1% (v/v) washed rabbit erythrocytes in PBS. The titer was read about 2 h later. The minimum detectable concentration of original toxin was in the range of 100 ng/ml.

Synaptosomes were prepared from rat brain according to Whittaker (1969).

All *dilutions* were made, if not otherwise stated, in a mixture consisting of 9 volumes of saline and 1 volume of 0.5 M sodium phosphate buffer pH 7.5 (PBS), with 0.1% (w/v) bovine serum albumin (Behringwerke, Marburg, trocken, reinst) added.

Results

1. Labeling of Botulinum Toxin

Crystalline botulinum toxin is known to consist of a neurotoxin and some hemagglutinins (DasGupta and Boroff, 1968). The material was scarce and the label could be used as an additional parameter during separation. Therefore we decided to label the crystalline toxin first and then to separate its iodinated components by gel filtration. Initial experiments with non-labeled crystalline toxin already had shown that the hemagglutinin peak is not homogeneous, since in any case the maximum of hemagglutination preceded that of protein (Fig. 1).

Labeling was done as previously described for tetanus toxin (Habermann, 1970) by aid of chloramine T (Greenwood *et al.*, 1963). Whereas tetanus toxin had always lost some toxicity on labeling (see Habermann, 1972), botulinum toxin tolerated it without measurable loss of activity.

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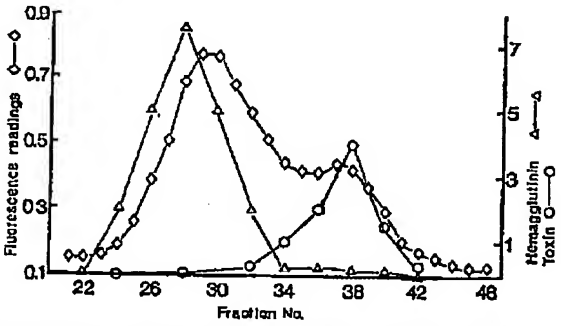


Fig. 1. Gel filtration of unlabeled botulinum toxin. Crystalline toxin (100 µg) was dissolved in 1 ml buffer solution (0.05 M Tris-HCl, pH 8.0, + 0.1 M KCl) and filtered at room temperature through a Sephadex G-200 column (1.5 × 76 cm) equilibrated with the same solution. 30 drops (i.c. 1.8 ml) per fraction were collected. Recovery was approximately 77% for the hemagglutinating activity, and approximately 36% with respect to the toxicity. Left ordinate: fluorescence (see "Methods") of the eluates. Right ordinate: activity equivalents (µg/ml of crystalline toxin)

Procedure. Carrier-free ^{125}I (corresponding to 2 mCi) in 0.05 M phosphate buffer pH 7.5 (100 µl) was kept with 100 µl chloramine T solution (0.1 mg/ml) in the same buffer for 1 min at room temperature. Then crystalline botulinum toxin (1 mg/ml in PBS, 100 µl) was added. After another minute, the reaction was stopped with 100 µl sodium metabisulphite (0.05 mg/ml) in phosphate buffer, and 100 µl of sodium iodide (20 mg/ml in water) prevented the non-specific adsorption of the label. The solution was made up to 1 ml with 500 µl Tris-HCl buffer as used for the gel filtration experiments (see Fig. 1).

In a typical experiment, 30.7% of the radioactivity proved to be precipitable with trichloroacetic acid. This would mean a specific overall radioactivity of about 6 mCi/mg protein. It should, however, be mentioned that neurotoxin represents only a fraction of it.—The toxicity remained unaltered (LD_{50} 2.5—5 ng/kg, mouse, s.c.). The same was rue for the hemagglutinating potency.

Upon gel filtration, the pattern of radioactivity (Fig. 2) approximately corresponded to that of the proteins (see Fig. 1). The hemagglutinating power (recovery 53%) was spread over a wide range of fractions which is at variance with the behaviour before labeling and indicates an alteration of the hemagglutinins. Toxicity, however, was well preserved (recovery 81%) and appeared at the expected place. Considering the overlapping between the neurotoxin and the main component of the crystalline material, the combined neurotoxic fractions where subjected to a second gel filtration under the same conditions. In order to avoid non-specific adsorption to surfaces, bovine serum albumin (0.1 ml of

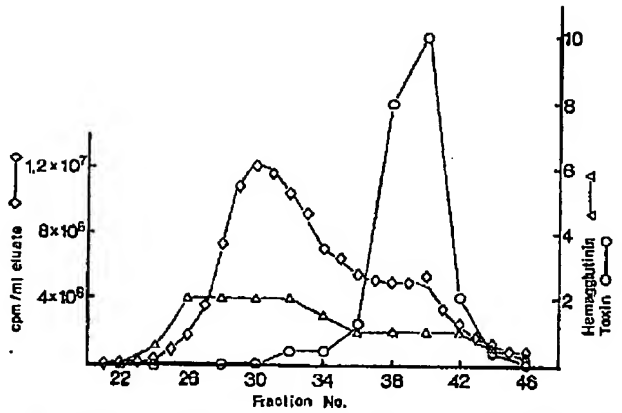
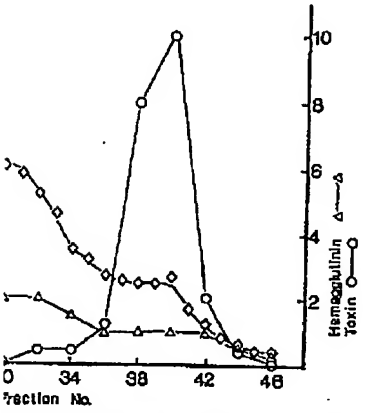


Fig. 2. Gel filtration of labeled botulinum toxin. The labeled (see p. 49) crystalline toxin (98 µg) was subjected to gel filtration as described in Fig. 1. The fractions 38—42 were combined and used for further experiments

a 1% solution in PBS) had been given into the collecting vessels. The resulting material (recovery of toxicity better than 50%), now homogeneous in gel filtration (Fig. 3), was stored in the frozen state. With respect to toxicity it was equivalent to about 1 µg/ml of the original crystalline toxin. Some label (up to 15%) may be detached during storage. It can be removed without loss in toxicity by overnight dialysis (Visking tubing) against PBS containing 0.1% sodium iodide and 0.1% sodium metabisulphite.

2. Binding of Labeled Neurotoxin to CNS Matter

The affinity of botulinum toxin A to CNS matter is still being disputed (see introduction) whereas that of tetanus toxin is beyond doubt. Thus we tested the labeled neurotoxin with brain matter and with isolated synaptosomes. Washed brain homogenate from rats fixed the toxin to a just measurable degree, although better than does liver homogenate. It cannot be excluded, however, that not the toxin itself but an accompanying labeled impurity has been bound. The binding becomes much more pronounced when the homogenate is replaced by synaptosomes prepared from rat brain (Table 1). The binding of the label can be prevented or reversed, in descending order, by botulinum antitoxin, botulinum toxin, or neuraminidase treatment (Table 2). Principally the same results have been obtained with tetanus toxin



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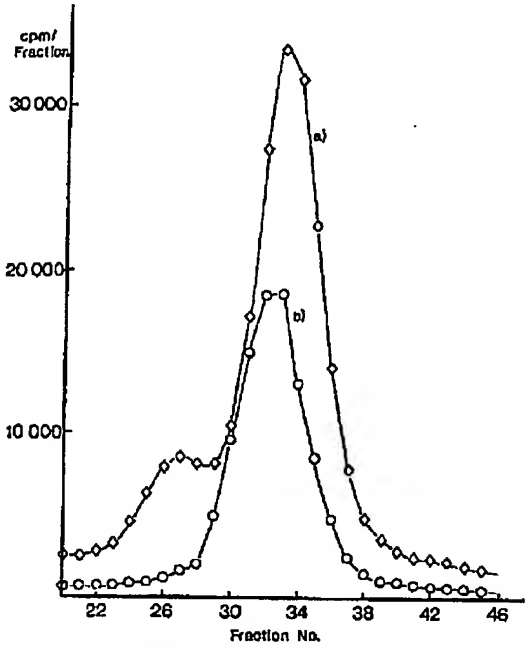

Fig. 3. Re-gelfiltration of labeled neurotoxin on Sephadex G-200. *a* An aliquot (0.2 ml) of the combined fractions Nr. 38—42 (see Fig. 2) was run through a Sephadex G-200 column (1×58 cm) prewashed with 0.5 ml 1% bovine serum albumin. 10 drops per glass (◇——◇). Note the impurity (arrow). *b* Purity control of the labeled neurotoxin (○——○) previously subjected to a re-gelfiltration. 0.7 ml of the combined fractions of the first re-gelfiltration (made under the conditions of Fig. 2) was applied to the 1×58 cm column

However, no significant binding of botulinum neurotoxin was found with ganglioside-cerebroside complex under the conditions described (Habermann, 1973) for tetanus toxin.

3. Ascent of Labeled Neurotoxin to the Spinal Cord in Rats

Tetanus toxin is known for its neural ascent into the spinal cord which process is crucial for the pathogenesis of local and probably also of general tetanus. Although the principally peripheral action of botulinum toxin might obscure any considerable role of neural ascent in botulism, we were interested to known whether the many similarities between the two toxins also refer to their behaviour within the CNS.

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Table 1. Dose-dependent fixation of labeled botulinum neurotoxin to synaptosomes. The labeled toxin, two times gelfiltrated and dialysed, was diluted 5 times in order to give a nominal concentration of 0.2 µg/ml. 0.05 ml of the labeled toxin solution (corresponding to 750 cpm) was mixed with 0.05 ml 0.5% albumin in PBS either with or without 5 µg unlabeled crystalline toxin. Then 0.2 ml of a preparation of brain synaptosomes (110 µg protein/ml in sucrose) was added and kept for 2 h at room temperature. Since the synaptosomes tend to agglutinate in salt solutions, they can be centrifuged off at 8000×g (Eppendorf centrifuge). 0.2 ml of the supernatant (a) and the remaining contents (c) of the vessels were counted for their radioactivities and the radioactivity bound (b) was calculated according to the formula $b = c - 0.5a$

Amount of synaptosomal protein added (µg)	Crystalline toxin added (µg)	% radioactivity bound
22	5	22.3
22	0	30.9
5.5	5	13.8
5.5	0	24.5
1.4	5	0.6
1.4	0	13.0
0.35	5	7.7
0.35	0	9.9
0.088	5	7.2
0.088	0	5.9
0 (sucrose)	5	6.4
0 (sucrose)	0	3.9

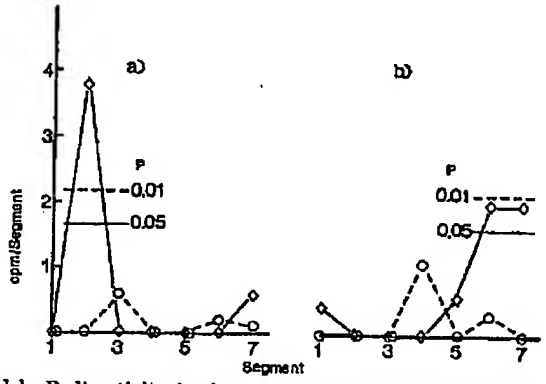

Fig. 4 a and b. Radioactivity in the spinal cord following application of labeled toxin with and without antitoxin. Ordinate: cpm/g WW in the respective spinal segments (1 cm length, numbered from caudal to cranial). —— $P = 0.05$; —— $P = 0.01$. a Toxin injected into the hind limb without (◇——◇) and with (○——○) antitoxin injected into the forelimb. b Toxin injected into the forelimb without (◇·····◇) and with (×——×) antitoxin injected into the hind limb. The animals were killed by exsanguination in ether anesthesia 24 h after toxin application

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al	Crystalline toxin added (µg)	% radioactivity bound
	5	22.3
	0	30.9
	5	13.8
	0	24.5
	5	0.6
	0	13.0
	5	7.7
	0	9.9
	5	7.2
	0	5.9
	5	0.4
	0	3.9

b)

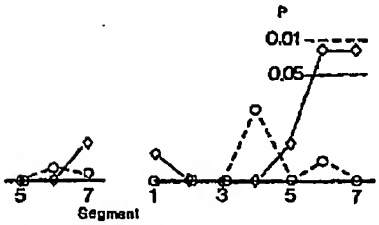

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Table 2. Prevention (a) and reversion (b) of binding of labeled botulinum neurotoxin to synaptosomes

a A suspension of synaptosomes (1 mg protein/ml; 0.2 ml) was incubated at room temperature with dilutions (0.05 ml) of unlabeled crystalline botulinum A toxin, or of botulinum antitoxin (Behring-Werke, Marburg; 750 IE/ml) made in PBS containing 0.5% bovine serum albumin. The neuraminidase (3.2.1.18; 500 E/ml, Behring-Werke, Marburg) was diluted in 0.05 M sodium acetate buffer, pH 5.5, containing 9 mg/ml NaCl and 1 mg/ml $CaCl_2$. 1 h later, a dilution (0.05 ml) of labeled botulinum A neurotoxin in saline, corresponding to 170 cpm was added, the mixture incubated for another hour, and centrifuged at room temperature (Eppendorf centrifuge, about 8000×g)

b Mixtures and incubations were as in (a); however, the radioactive solution was added first, and 1 h later followed by cold toxin, antitoxin, or neuraminidase. All incubations were performed at room temperature

Ingredient	Amount added	% bound	
		a (Prevention)	b (Reversion)
Toxin (cold)	5 µg	13.7	16.3
Toxin (cold)	0.5 µg	25.0	31.3
Toxin (cold)	0.05 µg	20.3	35.6
Antitoxin	3.75 U	0	5.0
Antitoxin	0.38 U	0	10.0
Antitoxin	0.038 U	16.6	30.5
PBS with albumin	—	31.7	34.1
Neuraminidase	75 U	7.0	13.3
Neuraminidase	7.5 U	7.7	18.6
Neuraminidase	0.75 U	29.3	35.3
Acetate buffer	—	33.8	36.8

Labeled, dialyzed botulinum toxin (about 0.2 µg/ml, corresponding to 8200 cpm/ml, 97.0% protein-bound radioactivity) was injected into either the left gastrocnemius or the left foreleg of rats in a volume of 0.2 ml/100 g body weight. In other animals, 0.1 ml/100 g antitoxin (Behring-Werke, Marburg) was injected into the crossed extremity. About 24 h later, the animals without antitoxin displayed massive local botulism. All animals were killed and their spinal cords removed. As shown in Fig. 4, injection of labeled toxin into the hind limb causes radioactivity to appear in the lumbar cord, whereas injection into the forelimb pushes some radioactivity into the cervical cord. Antitoxin given simultaneously into the crossed extremity prevents both processes. The amounts of toxin present in the nerves themselves were too small as to allow conclusions.

Discussion

Labeling of botulinum toxin is feasible with negligible losses in toxicity. Simultaneously with our work, Boroff and Shu-Chen (1973) labeled the purified neurotoxin of clostridium botulinum A with ^{131}I. In the workable range, at least 80% of the original toxicity had remained which is in good agreement to our findings with crystalline toxin. Botulinum A neurotoxin is of higher stability against iodination than tetanus toxin. The latter toxin always suffers losses as large as $^1/_2$ to $^3/_4$ of the original toxicity (see Habermann, 1972).

Gel filtration of crystalline toxin reveals that at least three different components were present. Hemagglutinating power appeared first and was followed by a non-identified fraction which accounted for most of the protein present. Toxin appeared as a shoulder at the decline of the latter peak and could be resolved by re-gelfiltration. After iodination, the hemagglutinin peak was considerably broadened and extended until the neurotoxin emerged from the column. It is still to be assessed whether the hemagglutinin is a protein fundamentally different from the main component of the crystalline toxin. Alternatively, the hemagglutinin might be composed of subunits, and its potency might depend on the number of subunits attached. This would explain why the maximum of unlabeled hemagglutinin appears at the high-molecular weight edge of the main peak. With the labeling process, the subunit structure might be disrupted, which should cause the hemagglutinating power to spread over a broad range of molecular weights. In any case, labeling the hemagglutinin did not give results as clearcut as obtained with labeled neurotoxin.

The binding studies reported are still incomplete. Nevertheless, it is already evident that botulinum toxin is able to interact with synaptosomes, and that this process may be partially reversed upon treatment with neuraminidase or antitoxin. Although central effects of botulinum toxin are of minor importance in botulism, binding to synaptosomes may become a useful tool for elucidating the mode of action of the toxin. Whether this is a specific fixation, a specific deactivation or a non-specific inactivation (van Heyningen and Mellanby, 1973), remains to be debated.

Botulinum toxin A shares its neural ascent with tetanus toxin, and some fixation seems to occur within the corresponding segments of the spinal cord. We do not know whether this is merely an oddity of the toxin or whether the neurotoxin exerts any spinal effects usually hidden by the peripheral ones. However, binding seems not to be a prerequisite of an action of the toxin. For instance, we did not find any accumulation of the labeled neurotoxin within the endplate region of the rat

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diaphragm bathed in toxin solution in vitro and autoradiographed thereafter (unpublished). It also could be questioned whether the spinal enrichment in vivo is, in the case of botulinum A neurotoxin, as specific as with tetanus toxin. Previously we injected labeled tetanus antitoxin in amounts exceeding, by weight and radioactivity applied, the amounts of botulinum neurotoxin used at present. No spinal accumulation took place, although the molecular weights of the immune globulins G and A are very close to those of the botulinum A and tetanus neurotoxins (Habermann, 1970). Even a non-specific interaction between botulinum toxin A and spinal cord would deserve special interest considering its extreme potency.

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